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Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes

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Nephrin is a slit diaphragm protein critical for structural and functional integrity of visceral glomerular epithelial cells (podocytes) and is known to be tyrosine phosphorylated by Src family kinases. We studied the role of phosphoinositide 3-kinase (PI3K), activated via the phosphorylation of nephrin, in actin cytoskeletal reorganization of cultured rat podocytes. Phosphorylation of rat nephrin by the Fyn kinase markedly increased its interaction with a regulatory subunit of PI3K. Stable transfection of rat nephrin in the podocytes with podocin led to nephrin tyrosine phosphorylation, PI3K-dependent phosphorylation of Akt, increased Rac1 activity, and an altered actin cytoskeleton with decreased stress fibers and increased lamellipodia. These changes were reversed with an inhibitor of PI3K and not seen when the nephrin-mutant Y1152F replaced wild-type nephrin. Rac1 and Akt1 contributed to lamellipodia formation and decreased stress fibers, respectively. Finally, in the rat model of puromycin aminonucleoside nephrosis, nephrin tyrosine phosphorylation, nephrin-PI3K association, and glomerular Akt phosphorylation were all decreased. Our results suggest that PI3K is involved in nephrin-mediated actin reorganization in podocytes. Disturbed nephrin–PI3K interactions may contribute to abnormal podocyte morphology and proteinuria.

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Visceral glomerular epithelial cells (GEC, also known as podocytes) play a central role in maintaining the selective filtration barrier of the renal glomerulus. Podocytes project numerous actin-rich processes called 'foot process.' Foot processes from adjacent podocytes form tight interdigitation and surround and support glomerular capillaries. Nephrin is a transmembrane protein, which belongs to the Ig superfamily and is localized at the slit diaphragm, which connects foot processes from adjacent podocytes.[1] Mutations of nephrin cause congenital nephrotic syndrome of the Finnish type;[2] thus nephrin has a pivotal role in glomerular permselectivity. Nephrin molecules from adjacent foot processes interact with each other in an antiparallel, homophilic manner, serving as a structural backbone of the slit diaphragm.[3] In addition to its structural role, research efforts in the recent years unraveled the importance of nephrin as a component of the slit diaphragm protein complex, which transmits signals into the cells.[4] The cytoplasmic domain of nephrin consists of approximately 150 amino acids and contains several tyrosine residues, six of which are conserved among human, mouse, and rat.[5] We and others have reported that the cytoplasmic domain of nephrin is tyrosine phosphorylated by the Src family kinase Fyn.[5–7] Tyrosine phosphorylation modulates the interaction of nephrin with other proteins such as another slit diaphragm protein, podocin,[5] adaptor protein, Nck,[8–10] and phosphoinositide 3-kinase (PI3K).[11] It is noteworthy that many of the nephrin-interacting proteins are known for their roles in actin regulation, suggesting the important role of nephrin in regulating the actin cytoskeleton and podocyte morphology.[12]

PI3K phosphorylates phosphatidylinositol lipids at the D-3 position of the inositol ring and converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate $PI(4,5)P_2$ (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate $PI(3,4,5)P_3$ (PIP_3).[13] PI3K consists of the two subunits, that is the regulatory p85 subunit (PI3K-p85) and the catalytic p110 subunit (PI3K-p110). The p85 interacts with phosphotyrosine containing motifs of activated growth factor receptors or adaptor proteins, bringing the p110 to the plasma membrane, the site of its enzymatic action.[13] PIP_3 generated by PI3K recruits pleckstrin homology containing proteins such as Akt and Rac guanine nucleotide exchange

factors (GEF) to the plasma membrane, initiating downstream signaling cascades.[13] Akt, generally considered as one of the main effectors of PI3K, is best known for its antiapoptotic/prosurvival actions.[14] In addition, a growing body of evidence indicates that Akt regulates the actin cytoskeleton and cell motility; Akt promotes the formation of lamellipodia and cell migration via its binding partner, Girdin, in Vero fibroblasts.[15] Similarly, activation of PI3K leads to decreased stress fibers and increased lamellipodia/filopodia via Akt and p70S6K in chicken embryo fibroblasts.[16] Akt also stimulates cell migration via modulating the interaction between Pak1 and Nck in HeLa cells.[17] A recent proteomics approach confirmed the direct interaction of Akt and actin in MCF-7 breast cancer cells.[18] In this study, the authors also demonstrated that the cortical remodeling of actin associated with cell migration was reversed by small interfering RNA directed against Akt. Furthermore, recent studies suggest the isoform-specific actions of Akt; Akt1-deficient cells showed increased stress fibers and decreased cell migration, whereas Akt2-deficient cells showed increased membrane ruffling and migration. Akt2 appeared to contribute to the inhibition of Pak1 and Rac1 as well.[19] Rac1 belongs to the Rho family of small GTPases. As many Rac GEFs (for example, Tiam1) are activated by PIP_2 or PIP_3,[20] Rac1 can also be activated by PI3K. Rac1 is one of the key regulators of the actin cytoskeleton in mammalian cells. In particular, Rac1 has been shown to mediate lamellipodia formation and membrane ruffling in response to growth factor stimulation.[20] In addition, Rac1 activity appears to be essential in regulating cell–extracellular matrix interaction and cell migration.[20]

A previous study by Huber *et al.*[11] showed that nephrin interacts with PI3K in a tyrosine phosphorylation manner, leading to the activation of Akt and increased cell survival. However, in this study, the precise mapping of phosphotyrosine-containing motifs in nephrin responsible for its interaction with PI3K was not reported. Also, potential consequences of PI3K activation other than cell survival were not addressed. In this study, we characterized a tyrosine phosphorylation-dependent interaction between rat nephrin and PI3K-p85 and focused on the impact of PI3K activation on the actin cytoskeleton. We demonstrated that nephrin–PI3K interaction leads to the activation of the Akt and Rac1 pathways, resulting in the remodeling of the actin cytoskeleton in cultured rat GEC.

RESULTS

Tyrosine 1152 is responsible for interaction of rat nephrin and PI3K-p85

We and others have reported that the cytoplasmic domain of nephrin is tyrosine phosphorylated by Src family kinases.[5–7] It was also reported that human nephrin interacts with PI3K in a tyrosine phosphorylation-dependent manner.[11] We first confirmed these results with rat nephrin. Wild-type, full-length rat nephrin and PI3K-p85 were transiently expressed in Cos-1 cells with or without the Src family kinase Fyn. Nephrin was strongly tyrosine phosphorylated only in the presence of Fyn (Figure 1a), consistent with our previous results.[5] Nephrin co-immunoprecipitated with PI3K-p85 in the presence but not in the absence of Fyn (Figure 1a). We also obtained similar results using the chimeric construct, in which the extracellular domain of the human interleukin-2 receptor is connected to the transmembrane/cytoplamic domain of rat nephrin (Tac/nephrin),[5] in the place of wild-type nephrin (data not shown). These results indicate that the cytoplasmic domain, but not the extracellular domain of nephrin, interacts with PI3K-p85 in a tyrosine phosphorylation-dependent manner.

To identify the binding site(s) for PI3K-p85, we analyzed the protein sequence of the cytoplasmic domain of nephrin by Motif scan analysis (http://scansite.mit.edu). This program identifies potential interacting proteins for various protein motifs in a given molecule. The results suggested that a motif containing Y1152 of rat nephrin (corresponding to Y1153 in mouse and Y1139 in human) is a likely binding site for PI3K-p85. Thus, we generated a nephrin Y1152F mutant and expressed it in Cos-1 cells with p85 and Fyn. In contrast to wild-type nephrin, the Y1152F mutant failed to co-immunoprecipitate with PI3K-p85 even in the presence of Fyn (Figure 1b). We showed previously that the Y1204F mutant of nephrin is tyrosine phosphorylated by Fyn significantly less than wild type and that its ability to interact with podocin is also significantly reduced.[5] Thus, for comparison, we studied the Y1204F mutant for its ability to interact with PI3K-p85. The Y1204F mutant co-immunoprecipitated with p85 in a Fyn-dependent manner similar to wild-type nephrin (Figure 1b). We have also tested other nephrin mutants including Y1127F (Figure 1b), Y1171F, and Y1194F (data not shown). All the mutants tested other than Y1152F co-immunoprecipitated with p85 in a Fyn-dependent manner. These results indicate that tyrosine phosphorylation of Y1152, but not the other tyrosine residues, plays a critical role in the nephrin (rat)–p85 interaction.

Nephrin activates Akt via PI3K

Nephrin is a transmembrane protein. Thus, by analogy to growth factor receptors, its interaction with PI3K-p85 is likely to activate the catalytic activity of p110, leading to the increased local concentration of PIP_3 and the activation of downstream signaling cascades such as the Akt pathway. To study whether nephrin activates Akt, we used cultured rat GEC and studied the activity of Akt using the antibody specific to the active form of Akt, which is phosphorylated at Ser473. In a subclone of GEC, which stably overexpress podocin and nephrin (GEC-P/N), nephrin was clearly tyrosine phosphorylated without any stimulation (Figure 2a). In these cells, phosphorylation of Akt was increased, as compared with GEC-neo (vector-transfected control; Figure 2a) or with GEC-P (overexpressing podocin alone; Figure 2b). Akt phosphorylation was not different between GEC-neo and GEC-P, suggesting that overexpression of podocin alone does not have an impact on Akt phosphorylation/

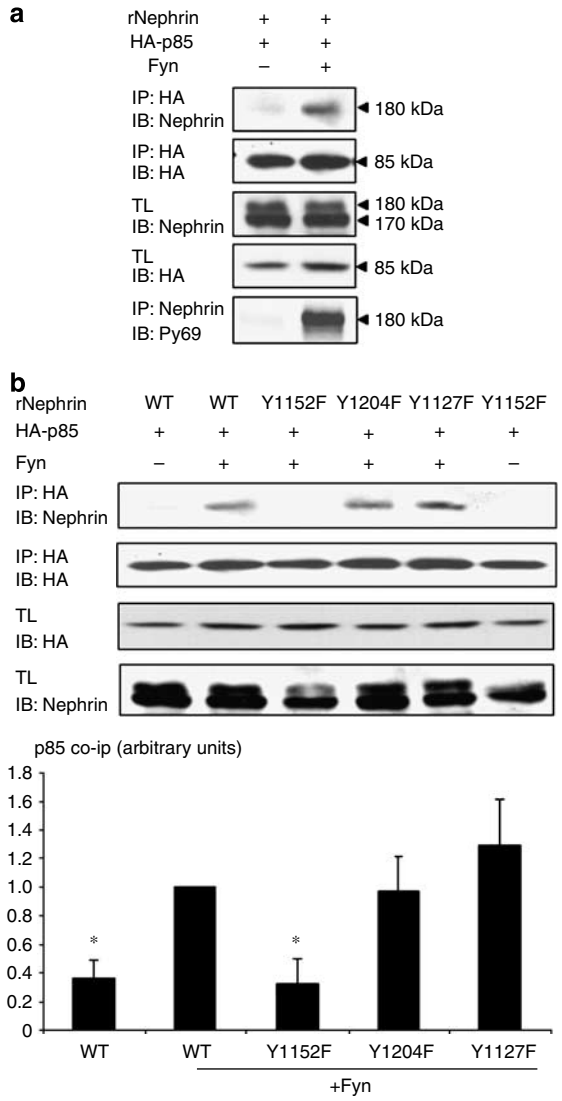

Figure 1 | Y1152 is responsible for tyrosine phosphorylation-dependent nephrin (rat)–p85 interaction. (**a**) Cos-1 cells were transient transfected with the indicated plasmids. After 24 h, cells were lysed and subjected to immunoprecipitation (IP). Precipitates were analyzed by immunoblotting (IB). TL, total cell lysates. Nephrin co-immunoprecipiated with PI3K-p85 only in the presence of Fyn. (**b**) Cos-1 cells were transiently transfected with the indicated plasmids and analyzed as in (**a**). Top: representative blots; bottom: densitometry. *$P<0.05$ vs WT with Fyn, $n=3$. Y1152F mutation, but not the other mutations of nephrin, abolished the interaction with PI3K-p85.

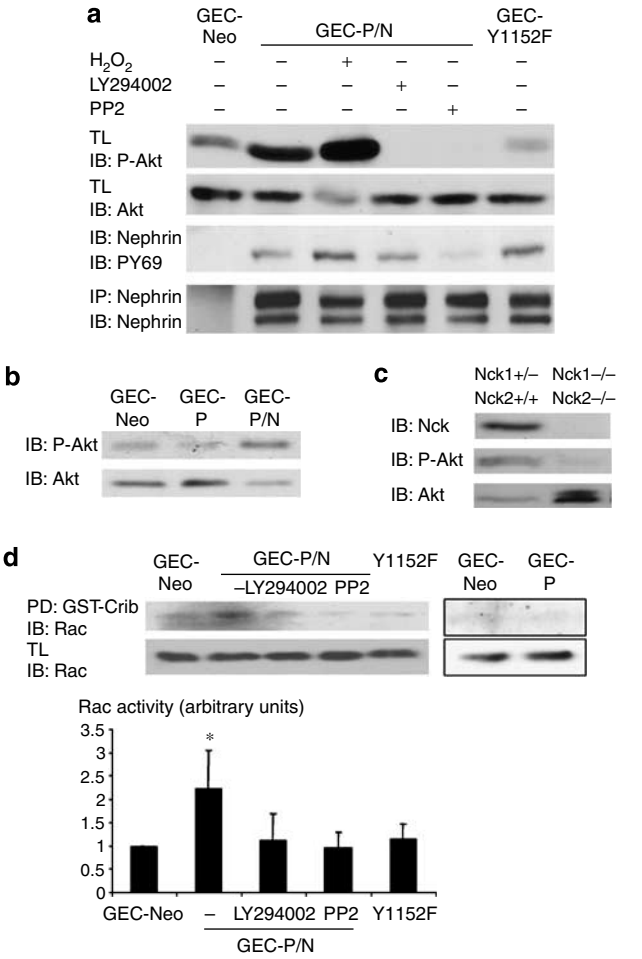

Figure 2 | Nephrin activates Akt and Rac1 via PI3K. (**a**) GEC were stably co-transfected with podocin and wild-type rat nephrin (GEC-P/N) or podocin and nephrin-mutant Y1152F (GEC-Y1152F). After 16 h of serum starvation, cells were treated with H_2O_2 (1 μM), LY294002 (PI3K inhibitor, 10 μM), or PP2 (Src family kinase inhibitor, 10 μM) for 30 min and were lysed and subjected to immunoprecipitation/immunoblotting. Akt phosphorylation was increased in GEC-P/N, as compared with GEC-neo (vector-transfected control cells) or GEC-Y1152F, which was abolished by LY294002 and PP2. (**b**) GEC were stably transfected with podocin alone (GEC-P). Akt phosphorylation in GEC-P was not different from GEC-neo and was less than that in GEC-P/N. (**c**) Akt phosphorylation was diminished in mouse embryonic fibroblasts (MEF) deficient in Nck1 and Nck2, as compared with Nck1 +/–, Nck2 +/+ MEF, under serum-starved conditions. (**d**) GEC-neo, GEC-P/N, GEC-Y1152F were serum starved for 16 h and LY294002 and PP2 were added 30 min before cells were lysed. Cell lysates were subjected to GST-Crib (Cdc42/Rac1 interaction binding) pull down (PD) followed by immunoblotting with anti-Rac1. Normalized to total Rac 1 and GEC-neo. Similar to Akt phosphorylation, Rac1 activity was increased in GEC-P/N. *$P<0.05$ vs GEC-neo, $n=3$–5. Rac1 activity was not different between GEC-neo and GEC-P (top right panel).

activation. Akt phosphorylation was completely inhibited in GEC-P/N by the PI3K inhibitor LY294002 and the Src kinase inhibitor PP2 (Figure 2a), suggesting that nephrin phosphorylates/activates Akt in a manner dependent on PI3K and Src family kinase(s). Stable expression of nephrin-mutant Y1152F, which lacks the ability to interact with PI3K-p85 in a tyrosine phosphorylation-dependent manner (Figure 1b), did not increase the phosphorylation of Akt (Figure 2a), supporting the role of PI3K in nephrin-mediated Akt activation. Additional clones of GEC-P/N and GEC-Y1152F were studied for Akt phosphorylation with similar results (Figure S1). In addition, antibody-mediated crosslinking of the extracellular domain of nephrin, a technique known to induce tyrosine phosphorylation of nephrin,[21] induced Akt phosphorylation (Figure S2). These results indicate that tyrosine phosphorylation of nephrin, most likely by Src family kinases such as Fyn,[5,7] activates the Akt pathway via PI3K.

Nephrin activates Rac1 in GEC

In addition to the Akt pathway, PI3K is known to activate the Rho-GTPase, Rac1.[13] Thus, we next studied Rac1 activity in various lines of GEC using a pull-down assay. The amount of active Rac1 was significantly increased in GEC-P/N, as compared with GEC-neo (Figure 2d). Rac1 activity was not different between GEC-neo and GEC-P, indicating that overexpression of podocin alone does not contribute to Rac1 activation (Figure 2d).

Similar to Akt phosphorylation, the increased Rac1 activity in GEC-P/N was inhibited by LY294002 and PP2 (Figure 2d). Furthermore, nephrin-mutant Y1152F failed to activate Rac1 (Figure 2d). Additional clones of GEC-P/N and GEC-Y1152F were studied with similar results (Figure S3). These data suggest that, in addition to the Akt pathway, nephrin activates Rac1 in a manner dependent on PI3K and Src family kinase(s).

Nephrin expression modulates the actin organization in GEC

The above results indicate that nephrin activates both Akt and Rac1 via PI3K. It was reported previously that nephrin-mediated PI3K/Akt activation leads to increased cell survival in GEC.[11] However, Akt is also known to regulate the actin cytoskeleton, which plays a central role in the morphology and function of GEC. In addition, Rac1 is a well-known regulator of the actin cytoskeleton. Therefore, we hypothesized that nephrin may modulate the actin cytoskeleton in GEC via PI3K-mediated Akt/Rac1 activation. First, we examined the pattern of F-actin in the cells by phalloidin staining (Figure 3). Control GEC (GEC-neo) showed well-defined cortical F-actin. Twenty-one percent of the cells demonstrated stress fibers, whereas 45% of the cells with free cell margin demonstrated membrane ruffles (or lamellipodia. These two terms are usually used interchangeably.[20]) In contrast, GEC-P/N showed cytosolic F-actin aggregates, whereas the cortical F-actin was less prominent. Stress fibers were observed only in ~4% of the cells, whereas a higher percentage of cells with free margin (~90%) demonstrated membrane ruffles. Decreased stress fibers and increased membrane ruffles observed in GEC-P/N were abolished by incubating the cells with LY294002 overnight. In striking contrast to wild-type nephrin, the Y1152F mutant of nephrin did not increase membrane ruffles nor did it decrease stress fibers. Rather, the number of stress fiber-positive cells

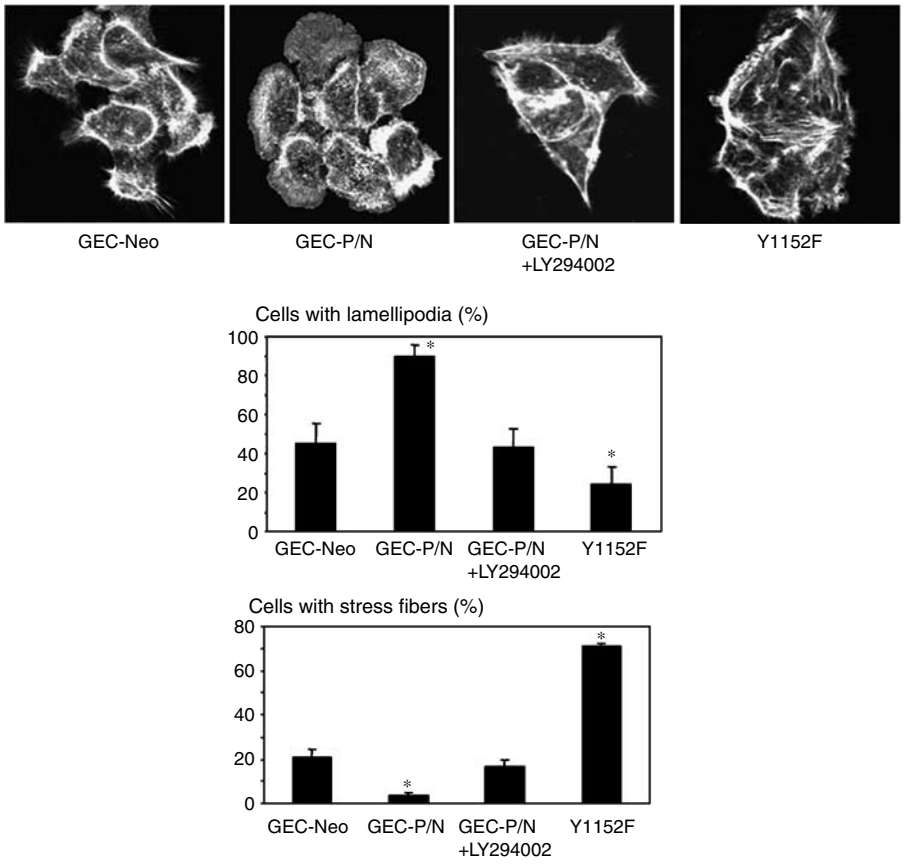

Figure 3 | Nephrin expression modulates the actin cytoskeleton in GEC. Serum-starved GEC-neo, GEC-P/N, GEC-Y1152F were stained with TRITC-phalloidin. LY294002 (10 μM) was added overnight. Cells with membrane ruffles and stress fibers were quantified as in Materials and Methods. *$P < 0.05$ vs GEC-neo, $n = 5$. GEC-P/N showed more membrane ruffles and less stress fibers, as compared with GEC-neo, and these changes were abolished by LY294002. Mutant nephrin (Y1152F) failed to induce these changes. F-actin pattern was not different between GEC-neo and GEC-P (data not shown).

increased significantly to 70% in GEC-Y1152F. The pattern of F-actin in GEC-P was not different from GEC-neo (data not shown). Additional clones of GEC-P/N and GEC-Y1152F were studied with similar results (Figure S4). Taken together, these results suggest that nephrin increases membrane ruffles/lamellipodia and decreases stress fibers in cultured rat GEC, and that these changes depend on PI3K.

Rac1 contributes to membrane ruffles in GEC

We next studied the role of Rac1 in the cytoskeletal changes observed in GEC-P/N. It is well known that Rac1 induces lamellipodia/membrane ruffles in fibroblasts.[20] Therefore, we hypothesized that Rac1 activated by PI3K contributes to increased membrane ruffles in GEC-P/N. First, we transfected CA-Rac1 in GEC-Y1152F, which had the lowest level of Rac1 activity (Figure 2d) and membrane ruffles (Figure 3) among various cells we established. Only 19% of the cells transfected with GFP alone showed membrane ruffles (Figure 4a), similar to untransfected GEC-Y1152F (23%, Figure 3). In contrast, most of the cells transfected with CA-Rac1 (82%) showed membrane ruffles. We next transfected GEC-P/N, which has the highest level of Rac1 activity (Figure 2d) and membrane ruffles (Figure 3), with DN-Rac1. Eighty-five percent of GEC-P/N transfected with GFP alone showed membrane ruffles (Figure 4b), similar to untransfected GEC-P/N (90%, Figure 3), whereas significantly less cells transfected with DN-Rac1 (61%) showed membrane ruffles (Figure 4b). Taken together, we concluded that Rac1 contributes, at least in part, to nephrin-mediated increase in membrane ruffles in GEC-P/N.

Akt1 decreases stress fibers in GEC

We next addressed the role of Akt activation in the cytoskeletal organization of GEC. It was reported previously that Akt1 decreases stress fibers in breast cancer cells,[22] whereas Akt1-deficient mouse embryonic fibroblast (MEF) shows increased stress fibers. To study if Akt1 also decreases stress fibers in GEC, we studied the impact of CA-Akt1 in

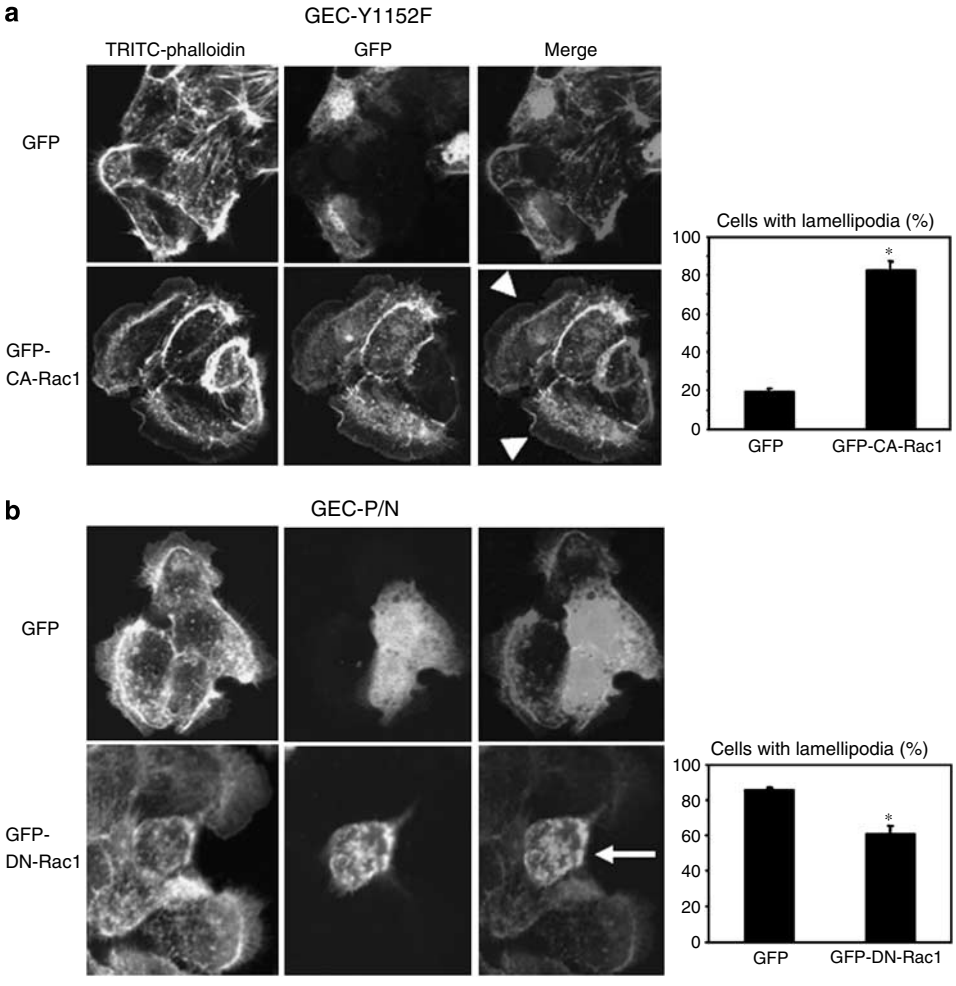

Figure 4 | Rac1 activation contributes to membrane ruffling in GEC. (**a**) GEC-Y1152F was transiently transfected with CA-Rac1 (GFP-tagged) or GFP alone. Cells transfected with CA-Rac1 showed prominent membrane ruffles at the periphery of the colonies (arrowheads). (**b**) GEC-P/N cells were transiently transfected with DN-Rac1 (GFP-tagged) or GFP alone. Cells transfected with DN-Rac1 were mostly devoid of membrane ruffles (arrow). Cells with lamellipodia were quantified as in Materials and Methods. *$P < 0.01$ vs GFP-transfected cells, $n = 3$.

cultured rat GEC. We chose to use GEC-Y1152F because these cells had low basal level of Akt activity (Figure 2a) and most cells had prominent stress fibers (Figure 3). Most GEC-Y1152F transfected with CA-Akt1 lost stress fibers, although such effects were not observed with GFP alone (Figure 5a). Similar results were obtained with GEC-neo, although the loss of stress fibers was less striking owing to the limited amounts of stress fibers in GEC-neo (Figure S5). We also confirmed these results using MEF deficient in Nck,[23] which have very low levels of Akt activity (Figure 2c) and prominent stress fibers.[8] Transient transfection of CA-Akt1 in Nck-null MEF decreased stress fibers significantly, as compared with cells transfected with GFP alone (Figure 5b). Taken together, these results support that Akt1 activation leads to decreased stress fibers both in GEC and in MEF. We also transfected GEC-P/N with DN-Akt1 to study if DN-Akt1 could increase stress fibers in these cells. However, we were unable to observe a clear increase of stress fibers by DN-Akt1 in these cells (data not shown, see Discussion).

Mechanisms of decreased stress fibers by nephrin

We further addressed the mechanism by which nephrin decreases stress fibers in GEC, focusing on the role of cofilin. Cofilin is an actin filament-severing protein and is inactive when phosphorylated. It is known that PI3K activates the cofilin phosphatase slingshot, leading to dephosphorylation and activation of cofilin.[24] Slingshot was colocalized with p-Akt in membrane ruffles, supporting the role of Akt in the activation of slingshot.[24] Thus, we reasoned that cofilin may be activated by nephrin via PI3K and Akt. We first compared

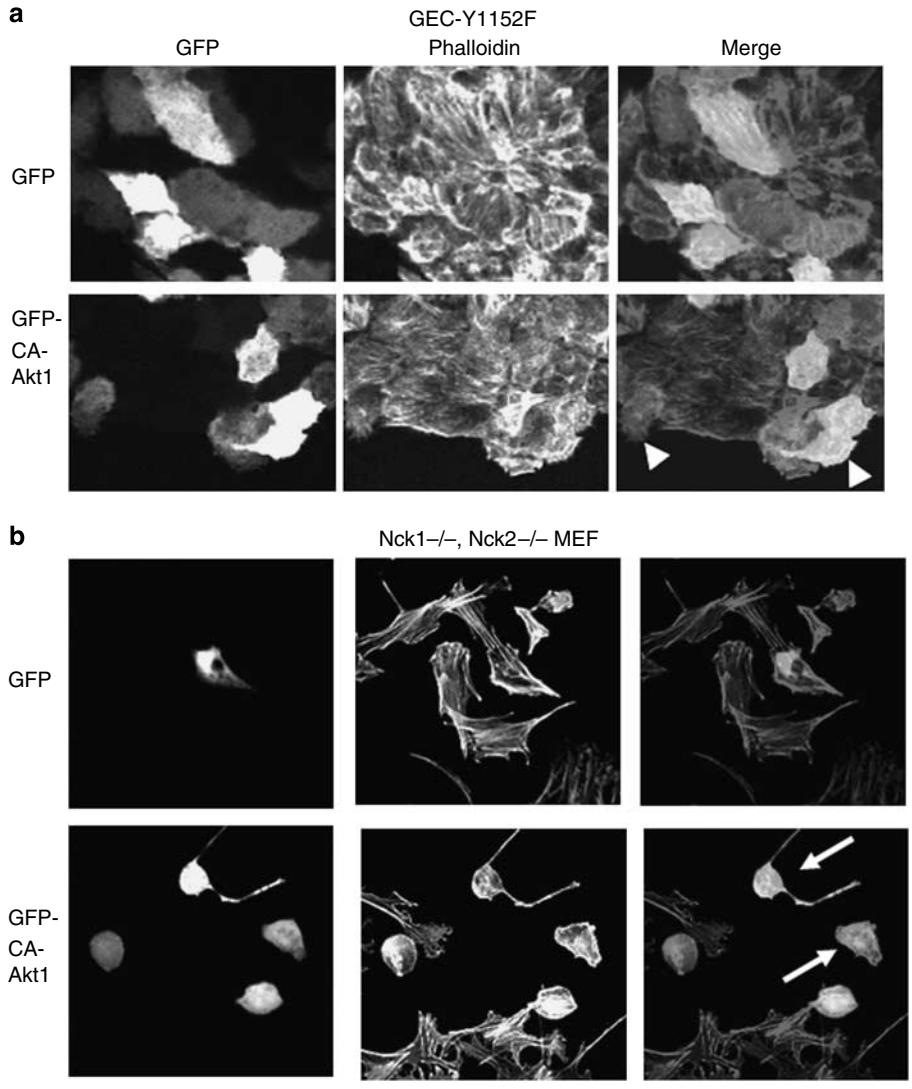

Figure 5 | Active Akt1 decreases stress fibers in GEC and MEF. (**a**) GEC-Y1152F, which demonstrate prominent stress fibers in ~70% of the cells (see Figure 3), was transfected with CA-Akt1 (GFPs-tagged) or GFP alone. Cells transfected with CA-Akt1 generally did not show prominent stress fibers (arrowheads). (**b**) For comparison, Nck-deficient MEFs (Nck1−/−, Nck2−/−), which have prominent stress fibers in ~100% of the cells and very low level of Akt phosphorylation (Figure 2c), were transiently transfected with CA-Akt1 (GFP-tagged) or GFP alone. Cells transfected with CA-Akt1 completely lost stress fibers and well-spread morphology (arrows).

cofilin phosphorylation in various GEC lines; cofilin was clearly phosphorylated (inactive) in GEC-neo, but its phosphorylation was decreased (more active) in GEC-P/N (Figure 6a). This decrease of cofilin phosphorylation was not observed in GEC-Y1152F (Figure 6a). We also showed that transient transfection of CA-Akt1 further decreases cofilin phosphorylation (activates cofilin) in GEC-P/N (Figure 6b). These results are compatible with the notion that PI3K activates cofilin via Akt, which may contribute to decreased stress fibers in GEC.

Another well-known regulator of stress fibers is the small GTPase, RhoA. When RhoA is activated, activation of LIM kinase via Rho kinase leads to phosphorylation and inactivation of cofilin, contributing to the stabilization of actin filaments.[20] RhoA also stimulates stress fiber formation via modulating the activity of a myosin light chain.[20] Therefore, we studied whether RhoA activity is regulated by nephrin. RhoA activity, quantified by a pull-down assay, was not different between GEC-neo, GEC-P, and GEC-Y1152F, but was consistently lower in GEC-P/N (Figure 7), corresponding to the level of cofilin phosphorylation and reciprocal to the changes in Rac1 activity. Of interest, when CA-Rac1 expression was induced in GEC, tyrosine phosphorylation of p190RhoGAP (GTPase-activating protein) was increased (data not shown), consistent with the previous report.[25] As the activity of p190RhoGAP corresponds to the level of its tyrosine phosphorylation, these results suggest that Rac1 activates p190RhoGAP, leading to inactivation of RhoA. This pathway may, at least in part, contribute to reduced RhoA activity in GEC-P/N, which, in turn, would contribute to decreased stress fibers in GEC-P/N.

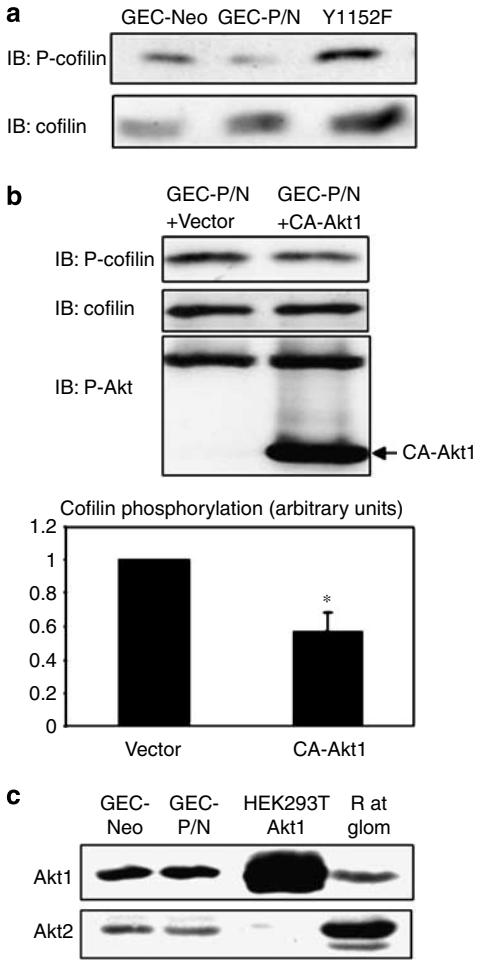

Figure 6 | Akt1 activates cofilin in GEC. (**a**) Serum-starved GEC-neo, GEC-P/N, and GEC-Y1152F were lysed and subjected to immunoblotting. GEC-P/N showed decreased phosphorylation (that is increased activity) of cofilin, as compared with GEC-neo or GEC-Y1152F. (**b**) GEC-P/N was transiently transfected with CA-Akt1 or vector. Top: representative blot; bottom: densitometric analysis. CA-Akt1 decreased phosphorylation (that is increased activity) of cofilin. $^*P<0.05$ vs vector, $n=3$. (**c**) Akt1 and Akt2 expressions in GEC and rat glomerulus. Lysate from GEC-neo, GEC-P/N, rat glomerulus, and HEK293T cells transfected with Akt1 were subjected to immublotting. Both Akt1 and Akt2 are expressed in GEC and rat glomerulus.

Akt activity is decreased in the rat model of PAN

We next used puromycin aminonucleoside nephrosis (PAN), a well-established rat model of podocyte injury and proteinuria,[26] to study the nephrin-mediated activation of the PI3K pathway *in vivo*. Nephrin was tyrosine phosphorylated in normal rat glomeruli and this phosphorylation was reduced in rats with PAN (Figure 8a), consistent with our previous reports.[5,8] In rats with PAN, there was a small but significant decrease in the amount of nephrin protein in total glomerular lysates (Control: 100 ± 1 (5 rats), PAN: 88 ± 7 (3 rats), arbitrary units, $P<0.05$). The amount of PI3K-p85 was not different between control and PAN. The amount of nephrin co-immunoprecipitated with p85 was decreased in PAN, as compared with control, and even after being normalized to the amount of nephrin in total lysates, this decrease was statistically significant (Figure 8b), indicating that the nephrin–p85 interaction is diminished in PAN. Akt phosphorylation was also decreased in glomeruli of rat with PAN, as compared with control rats (Figure 8c). As GEC is the major site of cell injury in PAN, it is reasonable to assume that this change in Akt phosphorylation occurs in GEC. Interestingly, similar changes were already observed at day 3.5, when proteinuria was either minor or negligible (Figure S6). These results indicate that in the PAN model of rat GEC injury, tyrosine phosphorylation of nephrin and nephrin–p85 interaction are reduced, likely contributing to decreased Akt activity in GEC.

DISCUSSION

Although it is widely acknowledged that the actin cytoskeleton is central to the normal morphology and function of podocytes, there is limited knowledge regarding how the actin cytoskeleton is regulated in podocytes. Recent report by Mundel and co-workers[27] highlighted the importance of synaptopodin in podocyte migration via indirectly regulating the stability of RhoA protein and modulating actin polymerization. These findings underscore the importance of the temporally/spatially coordinated collaboration of

various cytoskeletal regulators for podocytes to migrate to the appropriate location in the glomerulus, extend primary, secondary, and tertiary processes, eventually forming and maintaining the intricate morphology of the mature podocytes. Rac1 is a regulator of the actin cytoskeleton and is best known for its ability to stimulate lamellipodia formation in fibroblasts.[20] The lamellipodium is a membrane ruffling usually observed at the leading edge of motile cells. It is also observed at the site of directed outgrowth of the actin cytoskeleton, such as growth cones of axons, and the role of Rac1, in collaboration with the other Rho-GTPases, in neurite (including axon) outgrowth is well established.[28] In this study, we showed that nephrin-mediated Rac1 activation led to increased membrane ruffles in cultured rat GEC, accompanied by decreased cortical F-actin and stress fibers, most likely mediated by Akt activation (Figure 3). Proposed signaling pathways are summarized in Figure 9. Extrapolation of these findings to the *in vivo* setting requires caution, but we propose that disassembly of stress fibers/cortical F-actin by Akt activation liberates actin monomers necessary for actin remodeling and, combined with Rac1 activation, may lead to membrane protrusions, leading to cell migration and/or directional outgrowth, such as process formation. Conversely, decreased activities of Akt/Rac1 may contribute to foot process effacement. Notably, we did not observe obvious outgrowth of cellular processes in nephrin-expressing GEC. It is possible that diffuse expression of nephrin, as opposed to very specialized location of nephrin at the slit diaphragm *in vivo*, was not sufficient to stimulate directional cellular process formation. Alternatively, some key elements for process formation may be missing in cultured rat GEC used in this study. Obviously, the results obtained using the overexpression system need to be interpreted with caution. Unfortunately, however, there are no cultured podocyte lines to date, which reproduce genuine foot processes or the slit diaphragm seen *in vivo*.

Significance of stress fibers in cultured podocyte lines are still under debate. Podocyte foot processes *in vivo* are rich in actin filaments and foot process effacement, the hallmark of podocyte injury and proteinuric kidney disease, is often accompanied by the disappearance of these well-aligned

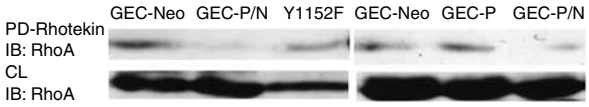

Figure 7 | RhoA activity is decreased in nephrin-expressing GEC. RhoA activity was compared by pull-down assay in GEC-neo, GEC-P/N, and GEC-Y1152F. RhoA activity was lower in GEC-P/N, but not in Y1152F, as compared with GEC-neo (left panel). RhoA activity was not different between GEC-neo and GEC-P (right panel).

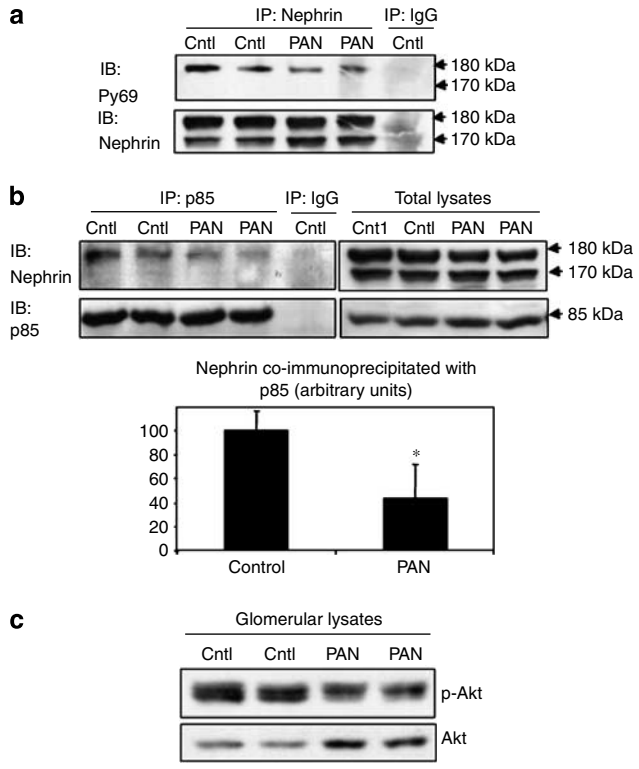

Figure 8 | Nephrin–p85 interaction and Akt phosphorylation are decreased in PAN. PAN was induced as in Materials and Methods. Glomerular lysates from rats with PAN (day 7) and control rats were immunoprecipitated for nephrin (**a**) or p85 (**b**) and precipitates were blotted for phosphotyrosine (**a**) or nephrin (**b**). (**a**) Tyrosine phosphorylation of nephrin was decreased in PAN. (**b**) The amount of nephrin co-immunoprecipitated with p85 was quantified by densitometry and normalized to the amount of nephrin in total lysates. $^*P<0.05$, $n=5$ rats for control, three rats for PAN. There was a small decrease (~12%) in the amount of nephrin in PAN (see text). The amounts of p85 were not different between control and PAN. (**c**) Akt phosphorylation was decreased in PAN.

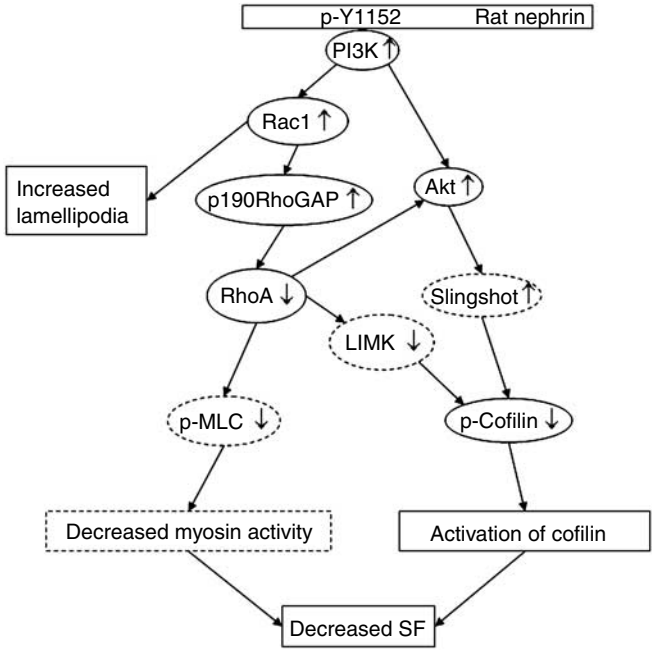

Figure 9 | Hypothesis for nephrin-mediated activation of the PI3K pathway and reorganization of the actin cytoskeleton. Dotted line represents the points not directly addressed in this study.

filaments.[29] Mundel *et al.*[30] established conditionally immortalized mouse podocytes, which could be differentiated, and showed that in differentiated cells, the actin cytoskeleton was rearranged into fibroblast-like stress fibers extending into the processes. Some investigators have assumed that stress fibers in cultured podocytes correspond to the filamentous actin in podocyte foot processes *in vivo* and as such represent differentiation of podocytes. However, stress fibers are generally observed only in cultured cells when cells make stable connections to substrates via focal adhesions and in this sense could be considered as an artifact of the cell culture system. Therefore, we suggest a view that stress fibers reflect various changes in intracellular signaling pathways and actin dynamics and are one of the useful tools in analyzing the behaviors of cultured podocytes, especially when combined with other markers. Nonetheless, by analogy to neurite outgrowth, it is possible that disassembly of stress fibers and formation of lamellipodia contribute to foot process formation in podocytes (as discussed above).

Nephrin-expressing GEC showed decreased stress fibers and increased membrane ruffles, as compared with control cells. In contrast, GEC overexpressing the Y1152F mutant of nephrin (which lacks binding to PI3K) showed significantly more stress fibers and less membrane ruffles, as compared with control cells (Figure 3). Within the scope of this study, we could not identify the cause(s) for the effects of nephrin Y1152F. We speculate that signaling pathways activated by this mutant nephrin are in favor of stress fiber formation, which becomes more visible when the counterbalancing force (PI3K/Akt1 activation via Y1152) is absent. Also, it was shown previously that effective membrane targeting of active Rac1 requires PIP_3;[31] thus in the cells expressing nephrin-Y1152F, Rac1 may not be properly targeted at the plasma membrane because of the lower activity of PI3K, leading to less membrane ruffles. These hypotheses need further verification.

On the basis of our hypothesis that nephrin-mediated Akt activation is a major contributor of stress fiber disassembly, we expected that inactivation of Akt would increase stress fibers in GEC-P/N. However, expression of DN-Akt1 in GEC-P/N did not have consistent effects on stress fibers. Recent studies have established isoform-specific actions of Akt on the actin cytoskeleton. Rat GEC used in this study and rat glomerulus express both Akt1 and Akt2 (Figure 6c). In MEF, Akt1 and Akt2 had opposing impacts on stress fiber formation.[19] As DN-Akt1 used in this study blocks both Akt1 and Akt2 by sequestering upstream stimulators, it is possible that the effect of Akt1 inhibition was masked by the simultaneous inhibition of Akt2. The phosphor-Akt (Ser473) antibody used in this study reacts with both isoforms; thus we were not able to discern which isoform(s) is/are activated by nephrin in GEC. Alternatively, there might be Akt-independent signaling pathways, which lead to stress fiber disassembly (see Figure 9).

In the rat model of podocyte injury and proteinuria (PAN), we observed that tyrosine phosphorylation of nephrin, as well as nephrin–p85 interaction and Akt phosphorylation, was decreased significantly (Figure 8). Although a causal relationship cannot be established in this model, it is reasonable to assume that impaired nephrin–p85 interaction contributes, at least in part, to decreased Akt phosphorylation. Because Akt is a well-known antiapoptotic molecule, these results are in line with the previous observations that podocyte apoptosis is often seen in PAN.[26] In addition, impaired nephrin–p85 activation may also contribute to deranged podocyte morphologies, such as foot process effacement.

MATERIALS AND METHODS

Materials

Tissue culture media and Lipofectamine 2000 were from Invitrogen Life Technologies (Burlington, ON, Canada). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON, Canada). Anti-nephrin antibody was described previously.[5] Anti-p85, anti-RhoA, anti-Rac1 antibodies, and rhotekin Rho-binding domain fused to GST (GST-RBD) were from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies for phospho-Akt (Ser473), Akt, Akt1, Akt2, phospho-cofilin (Ser3), and cofilin were from Cell Signaling (Beverly, MA, USA). Anti-HA antibody was from Zymed Laboratories (South San Francisco, CA, USA). Anti-Nck antibody and anti-phosphotyrosine antibody (PY69) were from BD Biosciences. Enhanced chemiluminescence (ECL) detection reagents and glutathione–Sepharose beads were from Amersham Bioscience (Baie d'Urfé, QC, Canada). TRITC-phalloidin was from Molecular Probes (Eugene, OR, USA). Male Sprague–Dawley rats were from Charles River Canada (St Constant, QC, Canada). Hydrogen peroxide was from Fisher Scientific (Lawn, NJ, USA). PP2, LY294002, puromycin aminonucleoside, and other chemicals were from Sigma-Aldrich (Mississauga, ON, Canada).

Plasmids

Plasmids encoding wild-type, full-length rat nephrin, rat nephrin tyrosine mutants, and Fyn were described previously.[5] pcAGGS-p85α encoding HA-tagged mouse p85 subunit was a gift from Dr Asano (Tokyo University, Tokyo, Japan). Mouse podocin cDNA was from Dr Benzing (University Hospital Freiburg, Freiburg, Germany). The plasmids pRK5-Rac1(L61)-Myc (constitutively active, CA), pRK5-Rac1(N17)-Myc (dominant negative, DN), and Cdc42/Rac interactive binding domain fused to GST (GST-CRIB) were from Dr Lamarche-Vane (McGill University, Montreal, QC, Canada).[32,33] Plasmid encoding CA-Akt1 (human) was from Dr Gotoh (University of Tokyo, Tokyo, Japan).

Cell culture

Rat GEC culture and characterization were described previously.[34,35] Briefly, GEC were cultured in K1 medium (50% DMEM (Dulbecco's modified Eagle's medium), 50% Ham F-12, 10% NuSerum, hormone mix) and studies were carried out between passages 10 and 60. GEC were stably transfected with pcDNA3.1 (GEC-neo), mouse podocin (GEC-P), mouse podocin, rat nephrin (GEC-P/N), mouse podocin and rat nephrin-mutant Y1152F (GEC-Y1152F). Nck-null (Nck1−/−, Nck2−/−) and Nck1+/−, Nck2+/+ MEFs were obtained from Dr Pawson (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada).[23] Transient transfection of Cos-1 cells and GEC was performed using Lipofectamine 2000 (Invitrogen Life Technologies).

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as reported previously.[5] Protein content was quantified using scanning densitometry (ImageJ software).

Induction of PAN and isolation of rat glomeruli

PAN was induced with a single intravenous injection of puromycin aminonucleoside (50 mg kg^{-1} body weight) in male Sprague–Dawley rats (150–175 g body weight) as described previously.[36] Rats were killed on day 7, when significant proteinuria was observed. Isolation of rat glomeruli was performed as described previously.[36] Studies were approved by the Animal Care Committee at McGill University.

Pull-down assays for active RhoA and Rac1

Preparation of GST-CRIB was described previously.[33] Cells or glomeruli were lysed in lysis buffer (25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.5), 1% NP-40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 5 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin). Equal amounts of protein (250–1000 μg) were incubated for 1 h at 4 °C with purified GST-CRIB or GST-RBD (10–15 μg) bound to glutathione–Sepharose beads. The beads and proteins bound to the fusion protein were washed twice and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and immunoblotting with anti-RhoA or anti-Rac1 antibody.

Immunofluorescence staining

Cells were plated on glass coverslips, fixed in paraformaldehyde (4%, 10 min, room temperature), permeabilized with 0.1%. Triton X-100, and blocked with 3% bovine serum albumin. F-actin was visualized by TRITC-phalloidin. Cells were examined by a confocal laser scanning microscope (Fluoview FV1000, OLYMPUS).

Quantification of lamellipodia and stress fibers

For the quantification of lamellipodia (membrane ruffles) in un-transfected GEC (Figure 3), at least 100 cells located at the periphery of the colony were counted per sample from randomly chosen fields. When $>50\%$ of the free margin of the cell was covered with lamellipodia, cells were considered positive for lamellipodium. For the quantification of stress fibers, at least 100 cells were studied per sample from randomly chosen fields, regardless of their location within the colonies, for the presence of clearly defined stress fibers. The percentages of lamellipodium- or stress fiber-positive cells were calculated for 5–7 samples and were averaged. For quantification of lamellipodia in GEC transfected with GFP-CA-Rac1, GFP-DN-Rac1, or GFP (Figure 4), only the successfully transfected cells, identified by green fluorescence, were counted. The other criteria were the same as in un-transfected GEC. For this series of experiments, at least 50 cells were counted per sample.

Data analysis

Data are presented as mean ± s.d. The *t*-statistic was used to determine significant differences between two groups. One-way analysis of variance was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the *t*-statistic and adjusting the critical value according to the Bonferroni method.

DISCLOSURE

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Nephrin activates Akt.
Figure S2. Nephrin crosslinking activates Akt.
Figure S3. Nephrin activates Rac1 activity.
Figure S4. Nephrin expression modulates the actin cytoskeleton in GEC.
Figure S5. Active Akt1 decreases stress fibers in GEC-neo.
Figure S6. Nephrin–p85 interaction and Akt phosphorylation are decreased in PAN.

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